In Vitro Stability and Pharmacokinetics of Novel Antileishmanial Compounds

A Senior Honors Thesis

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By

Kimberly N. Becker

The Ohio State University

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Project Advisor: Dr. James T. Dalton
Division of Pharmaceutics
College of Pharmacy

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I. Abstract

Leishmaniasis, a disease caused by protozoan parasites of the genus *Leishmania*, affects millions of people worldwide [6]. Without effective treatment, visceral leishmaniasis is associated with a near 100% fatality rate, whereas other forms can be severely debilitating [6]. Current treatments are not ideal because of toxicity, resistance, expense, and inconvenience [6].

Work to develop new drugs is underway at The Ohio State University and is led by Dr. Karl Werbovetz. Over the past few years Dr. Werbovetz and colleagues have generated a library of antiparasitic compounds known to specifically target *Leishmania* and trypanosome tubulin. In the first round of derivatization, several dinitroanilines were synthesized and evaluated in *in vitro* efficacy and stability and *in vivo* efficacy studies to identify key regions of the molecules for efficacy and metabolism. The most promising of these dinitroaniline compounds, GB-II-150, demonstrates an *in vitro* selectivity of two orders of magnitude for African trypanosomes over mammalian cell lines [7]. GB-II-150 was further evaluated in an *in vivo* metabolism study in rats [3] and found to be extensively metabolized with the major products resulting from N^1 ring oxidation, N^4 alkane oxidation, and N^4 oxidation [4]. Although GB-II-150 had a half-life of 170 minutes with intravenous administration, it was determined to be highly unstable with zero oral bioavailability when given via oral gavage [3]. Based on the results of these studies, analogs have been prepared in effort to achieve greater metabolic stability while maintaining selective antiparasitic activity [4].

A second round of derivatization and synthesis yielded another family of dinitroanilines that were again evaluated *in vivo* and *in vitro* for efficacy. My work has tested the *in vitro* stability and metabolism of several of these second generation dinitroaniline compounds that have shown antiparasitic activity. The most promising compound from this second round, TG-II-36, was also tested in an *in vivo* stability study to determine its pharmacokinetic properties. As a follow-up, ongoing work is being conducted on BTB-06237, an analog of a group of diphenyl thioether compounds that have also shown antiparasitic activity. This highly hydrophobic compound has presented serious challenges at early stages of analytical method development preventing adequate *in vitro* stability characterization.

II. Introduction

The leishmaniases are a globally widespread group of parasitic diseases caused by twenty species belonging to the genus *Leishmania*, a protozoa transmitted to humans primarily through the bite of the female sandfly [1]. This disease currently threatens 350 million people in 88 countries, mostly located in underdeveloped regions of the world [1]. Current treatment is expensive and available predominantly by injection or intravenous administration. Toxicity is a major concern for these therapies, as serious side effects and even death can occur. Recently, the first oral treatment for visceral leishmaniasis (Miltefosine) was registered in India [2]. The major concerns for this medication are cost and the potential development of resistance [2]. It is therefore imperative that an affordable, oral treatment is developed with high specificity for parasitic targets.

Tubulin is an established target in the chemotherapy of many diseases and is essential to all eukaryotes [5]. Tubulin is therefore a potential drug target for the treatment of many infectious diseases, including leishmaniasis [5]. As stated in a recent publication, ongoing work focuses on the stabilization of dinitroaniline and diphenyl thioether analogs to metabolic inactivation while maintaining antiparasitic potency and selectivity [5]. Although the diphenyl thioethers showed little activity, many of the dintroanilines have displayed *in*

vitro activity against African trypanosomes, and the most active antikinetoplastid compounds also inhibited leishmanial tubulin [5].

The primary goal of this research is to develop an orally active antileishmanial compound. A recent publication showed that the most promising compound for *in vitro* activity and metabolic stability (GB-II-150) among a group of synthesized dinitroanilines had zero oral bioavailability [3]. My work has paralleled this study with the *in vitro* stability characterization of a second generation of dinitroanilines followed by a pharmacokinetic study in rats to determine the disposition of the most promising of evaluated compounds (TG-II-36) [4]. In addition, I conducted experiments with BTB- 06237, a highly hydrophobic diphenyl thioether analog (Table 1), to better understand difficulties encountered in developing an analytical method for its quantitation.

III. Materials and Methods

LC/MS/MS Method Development:
Stock solutions (1 mM) of each dinitroaniline compound were prepared in DMSO. Stock solutions (1mM) of BTB-06237 were prepared in both DMSO and acetonitrile. Solutions (5 μM) were prepared in acetonitrile for tuning of mass spectrometry (MS) parameters. Once MS parameters were tuned, injections of each compound were made to evaluate chromatography peak shape and retention time. GB-II-143, a previously evaluated dinitroaniline, was used as an internal standard for all compounds.

Partial validation of TG-II-36 Analytical Method:
To partially validate the analytical method for quantitation of TG-II-36, multiple standard curves were generated in rat plasma and in neat solution. To cover the concentration range expected from the *in vivo* PK experiment, multiple standard curves were created with four replicates at concentrations of 100 nM, 1μM, and 10 μM in rat plasma for intra-batch and inter-batch accuracy and precision. Our method validation efforts were directed at measuring anticipated concentrations between 100 nM and 10 μM. However, higher than expected concentrations were observed after intravenous dosing of TG-II-36, and further validation at a higher range (100 μM) was conducted. This work was not completed, however, due to the disappointing rapid elimination and apparent toxicity observed with TG-II-36. Neat standard curves were prepared in 50%/50% (v/v) mixtures of acetonitrile and water to determine recovery.

***In vitro* Stability of Dinitroaniline Compounds:**
Stock solutions (1 mM) of each dinitroaniline compound were prepared. Standard curves were created covering 3nM to 1000nM with triplicates at 3nM, 30nM, and 300nM in final volumes of 330μL phosphate buffer with bovine serum albumin (BSA), pH 7.4. An aliquot (100 μL) of each solution was removed and added to 1mL of acetonitrile with 10nM GB-II-143. Tubes were then centrifuged and 1mL of supernatant was removed and placed in a new tube to be dried in the speed vac. For the S9 and BSA reactions, a 20mM solution of NADPH in phosphate buffer was created and a 6μM solution of the compound was added to each. At 0, 10, and 30 minutes 100μL of solution were removed from each tube and quenched with acetonitrile and 10nM GB-II-143. Tubes were centrifuged and the supernatant was removed and placed in the speed vac. All samples were reconstituted in 50%/50% (v/v) acetonitrile and water and placed in autosampler vials for mass spectrometric analysis.

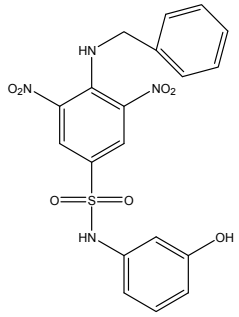
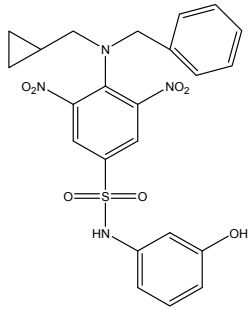
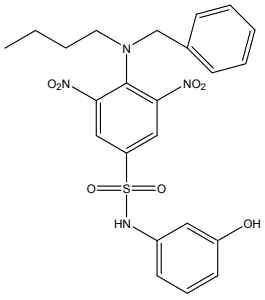
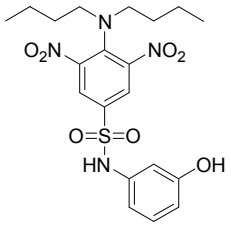
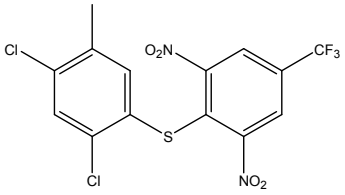
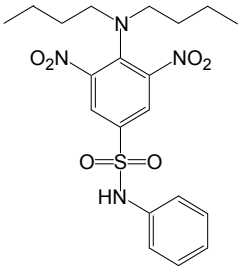
 TG-III-75	 TG-III-81	 TG-III-86
 TG-II-36	 BTB-06237	 GB-II-150

Table 1. Chemical structures of dinitroaniline and diphenyl thioether compounds evaluated

TG-II-36 *in vivo* pharmacokinetic study:

Based on its improved *in vitro* stability compared to GB-II-150, the pharmacokinetics and metabolism of TG-II-36 were further characterized. Male Sprague-Dawley rats (Harlan Bioscience, Indianapolis, IN) weighing between 270 g and 313 g were maintained in accordance with an animal protocol approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Animals were kept on a 12-h light/dark cycle with food and water ad libitum. One day before dosing, a catheter was implanted in the right jugular vein of each rat, and food (Harlan Teklad 22/5 rodent diet) was removed. The rats were supplied with water ad libitum and weighed immediately before dose administration. Food was returned 4 hr after dosing.

Twelve male Sprague-Dawley rats were randomly assigned to treatment groups and received TG-II-36 (MW = 466 g/mol) either intravenously or orally at a dose level of 10 mg/kg. Seven rats were dosed with TG-II-36 intravenously, four were dosed with TG-II-36 orally, and one was dosed with GB-II-150 (MW = 450 g/mol) intravenously. Dosing solutions of 10 mg/mL were prepared in 5% DMSO in PEG-300 (v/v). The jugular vein catheter was flushed with an aqueous solution of heparinized saline (100 U/mL, equal volume as the dosing solution and approximately three times the volume of the jugular vein catheter) immediately after administration of the intravenous dose. Serial blood samples (c.a., 120 µL) were collected at 2, 5, 10, 20, 30, 60, 90, 120, 240, 480, 720, and 1440 min after administration via the i.v. route, whereas serial blood samples (c.a. 120 µL) were obtained at 15, 30, 60, 90, 180, 240, 360, 480, 720, and 1440 min after dosing by oral gavage. Cannulas were flushed with 200µl

heparin/saline after blood draw. Plasma was separated by centrifugation (1000×g for 15 min at 4 °C), and samples were stored at −20 °C until analysis.

Rats were placed in metabolic cages individually when they were used in the pharmacokinetic study. Urine and feces specimens were collected at 12 and 24 h. All the urinary and fecal specimens were stored at −20 °C to protect against degradation.

BTB-06237 Analytical Method Development:

In addition to dinitroanilines, BTB-06237 was also selected for analysis due to its high antileishmanial activity with IC_{50} values of 0.5 ± 0.2 μM against *L. donovani* amastigotes [6]. This indicates higher potency than dinitroanilines previously tested in this assay [4]. BTB-06237 also showed selectivity by inhibiting the growth of Vero cells at higher concentrations (IC_{50} values of 5.3 ± 0.5 μM) [6]. A 1mM stock solution of BTB-06237 was prepared in acetonitrile for use in method development and *in vitro* stability. Initial studies were conducted to determine the linear range in neat and buffered solution and to evaluate stability in the S9 assay.

IV. Results

LC/MS/MS Method Development:

An analytical method was developed for each dinitroaniline compound using an Agilent 1100 LC system and a ThermoFinnigan TSQ Quantum Discovery Max Mass Spectrometer. Mobile phases consisted of 95%/5% (v/v) water/ACN (A) and 5%/95% (v/v) water/ACN (B), each containing 0.1% acetic acid. An Agilent Zorbax extended C18 column (2.1 x 50 mm, 3.5 μM) was used as the stationary phase. Eluents were monitored via electrospray ionization in negative ion mode with single reaction monitoring of the parent compound. Previously established gradient conditions of 50% B to 100% B between 0 and 1 minute, 100% B for 4 minutes, 100% B to 50% B for 0.1 minute, and 50% B for equilibration for the remaining 4 minutes of each run were used. Chromatographic separation was similar with all dinitroanilines, giving symmetrical peaks and elution times between 2.5 and 3.5 minutes. Linear ranges of quantitation between 3nM and 300nM were identified for each compound in phosphate buffer.

Partial validation of TG-II-36 Analytical Method:

A partial validation of the method for quantitation of TG-II-36 was completed in the range of 100 nM to 10 μM in rat plasma to assure accuracy and precision in the pharmacokinetic study results. Inter- and intra-batch accuracy and precision are shown in Table 2. Recovery of TG-II-36 was also determined as 63% when comparing peak areas in neat solution vs. plasma.

	Inter-batch (%)		Intra-batch (%)	
Calibration level	Precision	Accuracy	Precision	Accuracy
100 nM	11	13	17	3
1000 nM	17	10	8	6
10 μM	8	2	7	.1

Table 2. Intra- and inter-batch precision and accuracy for TG-II-36. Quintuplicate measurements were made in each experiment (n = 5) for inter-batch, and 3 experiments were completed (n = 15) for inter-batch calculations.

***In Vitro* Stability of Dinitroaniline Compounds:**

The *in vitro* stability of four dinitroaniline compounds (TG-III-75, TG-III-81, TG-III-86, and TG-II-36) was evaluated. These compounds were synthesized based on previous data suggesting that a *meta* hydroxyl group on the N1-phenyl ring maintains parasite selectivity, potentially by participating in hydrogen bonding with parasitic tubulin [4]. Results are displayed in Figure 1 and Table 3. Although these results indicated that TG-III-75 (the only secondary

amine tested) was the most stable, it is significantly less active compared to the other compounds [4]. TG-II-36 was moderately more stable than TG-III-81 or TG-III-86 and was chosen for *in vivo* evaluation.

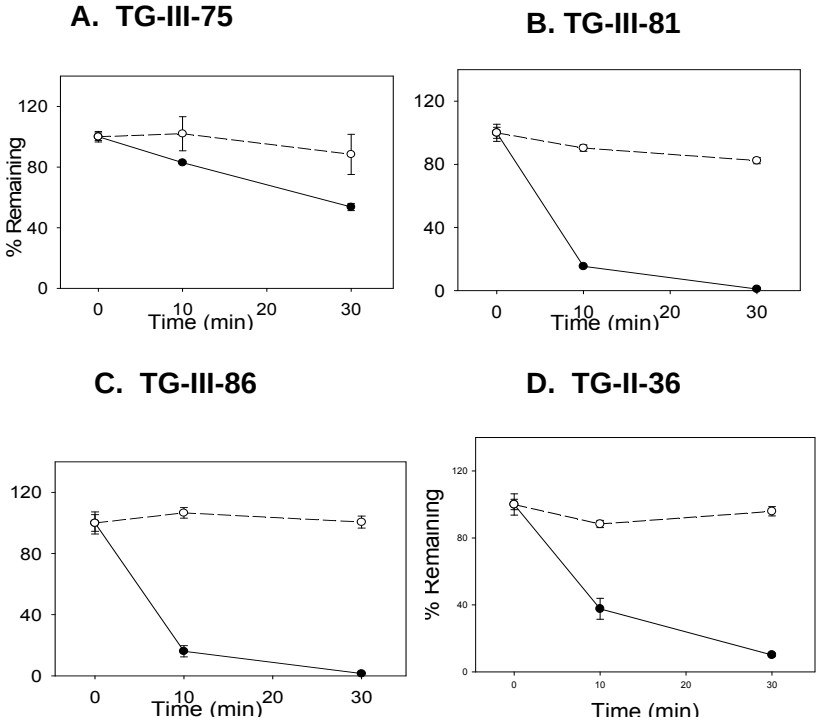


Figure 1: Preliminary *in vitro* stability results A) TG-III-75 B) TG-III-81 and C) TG-III-86. D) TG-II-36 at a final concentration of 300 nM were incubated with rat liver S9 (solid line; 1 mg/ml protein concentration) or bovine serum albumin (BSA, dashed line). The concentration of each compound remaining at each time point was determined using LC/MS/MS.

Compound	Percent remaining at 10 min[a]
TG-III-75	84 ± 7
TG-III-81	23 ± 2
TG-III-86	16 ± 3
TG-II-36	45 ± 5 [4]

Table 3. *In vitro* metabolism of compounds in the presence of rat liver S9
[a]Values represent means ± standard error of two triplicate experiments (n = 6).

TG-II-36 *in vivo* pharmacokinetic study:
TG-II-36 was poorly tolerated following intravenous dosing. One rat died during injection and two others died later (rat #4 died before the 4 hr time point and rat #5 died shortly after the 4 hr time point). Signs of hemolysis and/or renal dysfunction were noted in most animals, with red discoloration observed in the urine of rats immediately (animals #2, 3, and 5), at 30 min (animal #15) or at 2 hr (animal #1). Signs of hemolysis (i.e., red discoloration) were noted in the plasma

for animals that received TG-II-36 intravenously, but were absent from rats that received TG-II-36 rats via oral gavage. Similar but lesser signs of hemolysis were noted for animals that received GB-II-150, with discoloration only noted in the urine of animal #6. Animals #4, 7, 8, 9, and 14 also received GB-II-150, but produced normal urine. Further, hemolysis was only noted in the plasma for the later time points after intravenous administration of GB-II-150 rats. These results indicated hemolysis due to TG-II-36 and to a lesser extent GB-II-150. Rat #7 was dosed intravenously with 0.3 mL of the dosing vehicle (5% DMSO in PEG-300), 36 hrs after the original oral dose of TG-II-36, to determine if the vehicle was responsible for the observed hemolysis. Blood was taken 5 min after administration of the vehicle and plasma was clear. The rat reacted normally to the vehicle and the urine was unchanged (no hemolysis).

TG-II-36 was rapidly distributed and eliminated after intravenous administration to rats. Although plasma concentrations of TG-II-36 above 10 μM were observed in the early time points after intravenous administration, they declined below the limit of quantitation of the assay within four hours after dosing. The volume of distribution of TG-II-36 was approximately 1 liter/kg, suggesting that it is moderately bound by plasma and tissue proteins. The terminal elimination half-life for TG-II-36 was about 2 hours. Although the systemic clearance of TG-II-36 was 16.6 mL/min/kg, or about one-half of that previously reported for GB-II-150 [3], the mean area under the plasma concentration-time curve (AUC) for TG-II-36 in four rats was approximately equal to that observed for a single rat that received GB-II-150 in the current study. The reason for the observed difference in GB-II-150 pharmacokinetics between the current and previous study is unknown. The high clearance of TG-II-36 suggests that it undergoes extensive hepatic metabolism and extraction. Accordingly, plasma concentrations of TG-II-36 were detectable but below the limit of quantitation after oral administration to rats.

Parameter	**GB-II-150 previous data**	**GB-II-150**	**TG-II-36 NCA**
$t_{½\beta\ (terminal\ half\ life)}$ (min)	170	42	124 ± 7.6
AUC (min μg/mL)	285 ± 69	620	713 ± 213
CL (mL/min/kg)	31.5 ± 7.0	15.9	16.6 ± 4.2
V_{ss} (mL/kg)	4981 ± 1757	539	981 ± 433

Table 4. Pharmacokinetics of GB-II-150 and TG-II-36 after intravenous administration of 10 mg/kg, values are mean ± S.E. (n=4) except GB-II-150 where only one rat was used. Noncompartmental analysis (NCA) was used for all GB-II-150 rats (Wu, et al., 2006).

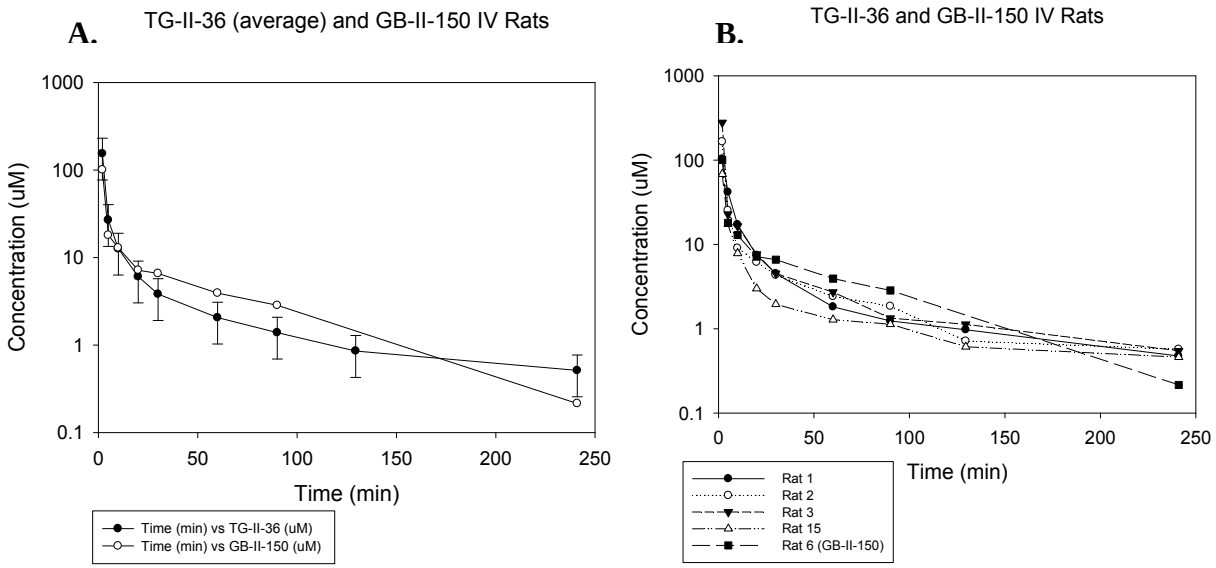


Figure 2
(A) Average concentrations for four intravenous TG-II-36 rats and one GB-II-150 rat ranging from 2-240 min. All other time points (480-1440 min) were below the quantitation limit and the 240 time point for the GB-II-150 rat was below the quantitation limit. (B) Concentrations for all intravenous TG-II-36 rats and one GB-II-150 rat ranging from 2-240 min.

BTB-06237 Analytical Method Development:

Evaluation of BTB-06237 in the S9 assay produced poor results. With a concentration range between 3 nM and 300 nM, the same range used for the aforementioned dinitroaniline compounds, linearity was not consistently observed for BTB-06237 in neat solution, and it was undetectable in S9 reaction buffer. Additional experiments were therefore added to understand the difficulties encountered with the use of this compound in our existing metabolic stability assays.

Aqueous solubility was suspected as a potential cause for the poor sensitivity observed in standard and reaction samples. An early study was conducted to evaluate the use of Pluronic F-127, a polymer that had shown promise for increasing BTB-06237 solubility in water, to determine its utility in method development and S9 reactions. However, results with this polymer were indistinguishable when compared to the control without F-127 (data not shown). A study was therefore conducted to compare BTB-06237 signal intensity in various solvents to assure stock solutions, reaction solution, extraction and reconstitution solvents were appropriate. Figure 3 displays the results from this study. Although acetone provided the highest signal for BTB, good signal intensity was observed for all organic solvents tested. BTB-06237 produced very low signal in water, indicating potentially poor solubility and possible precipitation. However, given that aqueous solubility was apparently adequate for IC_{50} determinations during *in vitro* efficacy assays in the micro-molar range [6], we evaluated the use of higher concentrations for quantification. Results were mixed as we identified a linear range at these higher concentrations, but signal intensity was very low in S9 phosphate reaction buffer compared to neat solution. Figure 4, plot 1 displays the linearity and chromatographic peak areas achieved between 300 nM and 30 μM in neat solution. The remaining plots indicate the lower signal in phosphate buffer (plots 2A and 2B) and water (3).

Further experiments were conducted at the higher concentration range to better understand the causes for poor signal intensity in aqueous solution. These studies focused on solubility, stability and matrix ion suppression effects. These experiments included the

evaluation of sample vial (glass vs. plastic), addition of vitamin C to stabilize BTB-06237, and stability during processing (extraction, drying and storage). Figure 4, plots 2A, 2B and 3, illustrate results from one of these studies. When phosphate is included in the sample (plot 2A), some signal suppression is observed compared to water alone (plot 3) indicating a slight matrix effect. This plot also illustrates that some signal loss occurs when comparing before (plot 2A) and after sample drying in the vacuum evaporator (plot 2B). Further work is planned to evaluate the time required for BTB-06237 to dissolve in water as a potential source of variation in our assays. BTB-06237 has proven difficult to work with *in vitro* due to an apparent low solubility or stability in aqueous environments (buffer and/or plasma).

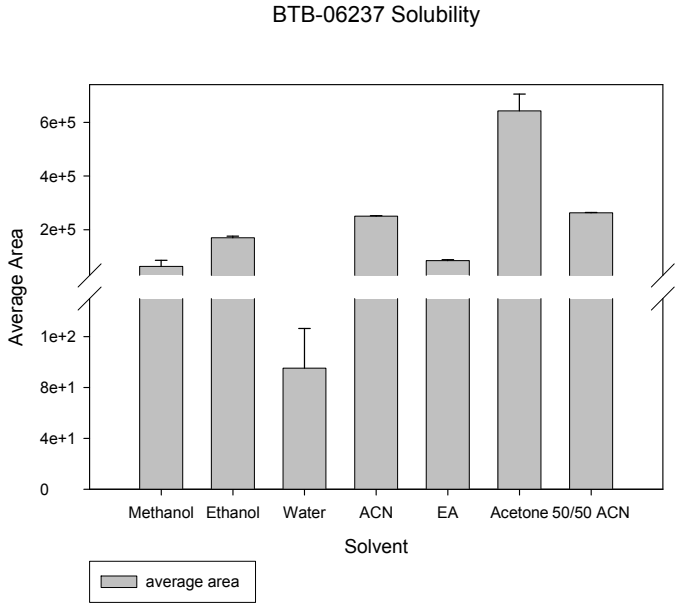


Figure 3. 1μM solutions of BTB-06237 in various solvents: BTB-06237 (1mM in DMSO) was added to a each solvent to produce a 1μM solution. Samples were injected into the LC/MS/MS method for separating and detecting BTB-06237, and chromatographic peak areas (y-axis) were observed for comparison.

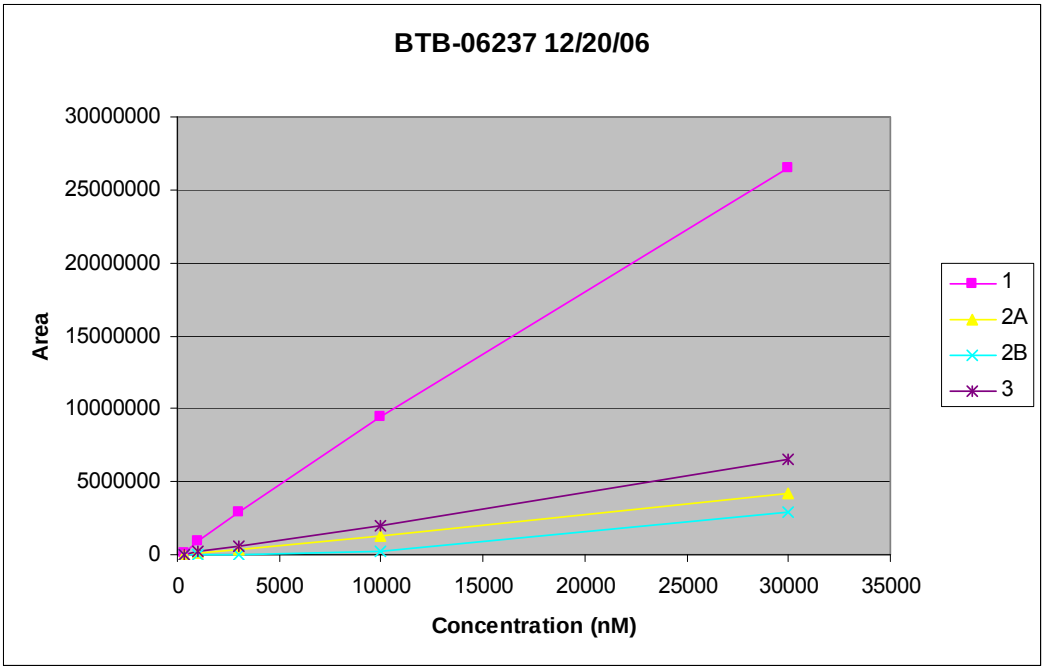


Figure 4. Standard curves of BTB-06237 in neat solution (1), phosphate buffer (2A and 2B), and water. Signal loss was evaluated by adding BTB-06237 at different steps in the sample preparation process. As an example, plot 2B indicates some signal loss during the drying process in the vacuum evaporator (compared to 2A). Plot 2A indicates a potential matrix effect when compared to plot 3 (phosphate buffer vs. no buffer). (y-axis is chromatographic peak area).

V. Discussion and Conclusion

We successfully developed accurate and sensitive LC/MS/MS methods for the quantitation of several novel dinitroanilines. We used these methods to study the *in vitro* stability of four compounds in the presence of bovine serum albumin (protein control) and hepatic S9 fractions from the rat liver. Pilot studies to develop an analytical method for a diphenyl thioether, BTB-06237, were also conducted. Lastly, we evaluated the *in vivo* pharmacokinetics of one dinitroaniline, TG-II-36, after intravenous and oral dosing to rats.

The pattern of N4 substitution had little to no influence on the *in vitro* metabolic stability of the four dinitroanilines studied; with the exception that the one secondary amine tested (TG-III-75) was the most stable and least active of the compounds tested. TG-II-36 was the most stable of the remaining three compounds, but was also extensively metabolized during *in vitro* incubation with S9 hepatic fractions (i.e., only 45% remaining at 10 minutes).

Pharmacokinetic studies of TG-II-36 showed that it was moderately distributed and rapidly cleared after intravenous administration, while oral dosing studies demonstrated that less than 5% of the dose appeared in the systemic circulation after oral gavage. We therefore conclude that TG-II-36 has limited oral bioavailability. When compared to GB-II-150, TG-II-36 appears to be more toxic. Three rats died during the course of the pharmacokinetic studies. Almost all intravenous rats experienced hemolysis. Rats injected with GB-II-150 experienced slight hemolysis during a previous study (Wu et al., 2006), but it was not as severe or as rapid onset as the rats injected with TG-II-36 in our study. Although TG-II-36 appeared to be more stable *in vitro* than GB-II-150, it appeared to have comparable stability *in vivo*.

The class of diphenyl thioether compounds, including BTB-06237, do not seem to target tubulin and do not work in the previous dinitroaniline *in vitro* stability assay. Our pilot studies

show that the type of tubes used (plastic vs. glass) do not have a significant affect on the mass spectrometry results. It is possible that some of the compound may be degraded during sample processing or that it is unable to be fully detected upon LC/MS/MS analysis because of matrix effects. Further work with these compounds may prove to be very difficult given solubility and analytical method development issues. Work with this compound and others will continue in the lab.

VI. Acknowledgements

This research project was conducted under the auspices of a collaborative project between my advisor (Dr. Jim Dalton) and Dr. Karl Werbovetz. I would like to thank Dr. Jim Dalton, Dr. Karl Werbovetz, members of the Werbovetz and Dalton labs, Dr. Mitch Phelps, and Dr. Duxin Sun. Undergraduate Research Scholarship, Summer Undergraduate Fellowship, and NIH grant AI062021 (to KAW).

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